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Isolation of *Ascochyta lentis* fungus, responsible for blight disease in lentil crop and screening of suitable fungicides for management

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Lentil is an important legume crop that is a safe source of high-quality dietary protein. Mostly fungal diseases are considered an important constraint of Lentil. Blight disease caused by Ascochyta lentis is an important disease caused by *Aschochyta lentis*. Samples from diseased plants were collected, and the pathogen was isolated on artificial media. After purification, the pathogen was indentured based on the growth pattern, colony shape, and spore structure. There is very fewer germplasm of Lentil available in the country. The current study shows 12 lentil genotypes (11504,12512,12514, M-09501, Pb-m-2009, M-93,12505,12503,11507,11509,10503, M-85) were screened against Ascochyta lentis by following RCBD under natural and artificial disease conditions. Agronomic, morphological and physiological data were recorded from field trials. Fungicides were diluted into different concentrations and were used to inhibit the growth of A. lentis under in-vitro conditions. Similarly, BCAs were also screened against A lentis on artificial media plates and were utilized in field conditions to manage the disease.
Keywords: Ascochyta lentis, Biocontrol agents, Disease resistance, and Lentil blight.

INTRODUCTION

Lentil (*Lens culinaris* L.) is one of the oldest legume crops cultivated in South West Asia, about 7000 BC. (Ahad and Matiur, 1993). Most lentil crop is grown in Asian countries. Worldwide, it is cultivated on an area of 4600000 hectares and gives 4200000 tons of seeds (FAO, 2016). Pakistan is the major lentil producer in south Asia. It is grown on 33,909 hec and 17909 tons of production. (Agriculture Statistics of Pakistan, 2019). Lentils are rich in nutrition which is beneficial for human health. They have the highest protein content, at 30% of their calories. It also has a great source of carbohydrates and fiber. Moreover, they contain important minerals and vitamins like iron and zinc (Callaway, 2004). Lentil contains 28.6 percent protein, 3.1 percent ash, 4.6 percent crude fiber, 44.3 percent starch, 36.1 percent amylose, and 63.1 percent total carbohydrates, providing 420 Calories per 100 gm (Yadav *et al.*, 2017). Moreover, lentils are lower in anti-nutritional factors such as hemagglutinins, oligosaccharides, and flavones than most other legumes. Lentils contain tannins in the seed coat but not in cotyledons, and their consumption is safe as they are used in the human diet after the removal of the seed coat (Srivastava and Vasishtha, 2012). Lentils have two types: large-seeded Lentils called *macrosperma* and small-seeded *microsperma*. The *macrosperma* (large-seeded lentils) contain tannins, which can cause digestive disorders. The high protein level, a lower level of anti-nutritional factors, and a shorter cooking time compared with most other pulses make Lentils very suitable for human consumption. Ascochyta blight of Lentil was first discovered in 1938 (Bondartzeva-Monteverde and Vassilievsky, 1940). It has been reported in many lentil-growing areas (Vandenberg, 2009). Gossen *et al.* (1986) proposed that *Ascochyta lentis* should be synonymized with *A. fabae*. Ascochyta blight (AB) of Lentil caused by *A. lentis* Vassilievsky (teleomorph: *Didymella lentis*) is prevalent in many lentil production regions of the world and has been reported to cause yield losses up to 70 %, 30-50 % and 50 % in Canada, USA and Australia, respectively (Brouwer *et al.*, 1995). Morrall and Sheppard (1981) described isolates of *A. lentis* and reported that generally, A lentis fungus produces 2-celled conidia of 10-20 × 4-8 μm (Mean: 15.8 × 5.7 μm) with a few multi-septate conidia among a majority of 2-celled forms. The taxonomy of *A. lentis* was a topic of discussion in the 1980s when it was initially considered a forma specialis of A. fabae because of high microscopic and physiological

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resemblances (Gossen and Morrall, 1984). In 1993, *Didymella lentis*, the teleomorph of *A. lentis*, was found on overwintered lentil debris in the Palouse region of eastern Washington and northern Idaho. Kaiser and Hellier (1993) differentiated the teleomorphs of *A. fabae* and *A. lentis* based on pathogenicity tests and morphology. Their pycnidia release conidia which move short distances via rain splash and wind. A. lentis is heterothallic with two typical mating types (MAT1-1 and MAT1-2) (Falahati *et al*., 2010). Conidia infect lentil plants following germination at the optimum temperature of 20-25°C and in the presence of free moisture. The pathogen survives between lentil crops on or inside the seeds, on crop residue and volunteer lentil plants. Ascochyta blight, caused by *Ascochyta lentis*, is an important disease of Lentil. The disease severely affects seed quality and yield (Iqbal and Mukhtar, 2014). Blight disease is an important economic concern in Australia, Canada, India and Pakistan (Ilyas *et al*., 2010). Gossen and Morrall (1983) reported that severe foliar infection resulted in yield losses of over 40%. Similarly, Hussain *et al.* (2016) estimated that 30-40% of crop losses in Pakistan. In some cases, seed infection is so severe that the lentil seeds lose their quality and become less attractive in the market. In the present study, isolation and identification of the fungal pathogen associated with lentil blight were performed Chemicals were only sorted out in the *in-vitro* as fungicide application on spores to find the suitable dose. While BCAs were tested in dual plates assays to find the potential Biocontrol agent and, later, were applied in the field to manage the disease. In the field trials, fungus pressure was managed using BCAs while creating disease in the lentil genotypes compared to untreated control plants.

MATERIALS AND METHODS

Isolation of Fungus: Infected plants were collected from different areas and brought into Lab. Plant samples were disinfected with 70% ethanol and placed on PDA media. The samples were incubated for 20 days at 22±2°C. After three days, the fungus was purified by picking a hypha tip and placed on fresh media plates. Purification of the pathogen was done by mycelium transfer and hyphal tip method. The fungus was identified using available literature, especially based on plate color, colony pattern, conidia, spore shape, size and structure by slide preparation for microscopy (Keogh *et al.,* 1980). After one week, spores were harvested using chilled water, followed by sieving through four layers of sterile muslin cloth. Spores of *A. lentis* were counted by adding 1µl inoculum on a hemocytometer under a microscope. The inoculum was adjusted to 10^5 spores/ml suspended in distilled water.

***In-vitro management of A. lentis*:** In-vitro management of A. lentis was done by using commercially available fungicides. For this, commercially available chemicals (Tropsi M, Segawin, difenoconazole, Sulphur, thiomyl, dolomite, cymoxinal mancozeb and metalaxyl mancozeb) were diluted to 250mg/ml, 125 mg/ml, 62.5 mg/ml 31.2 mg/ml 15.6 mg/ml 7.8 mg/ml 3.9 mg/ml in distilled water and tested as 50 µl against 50 ul spore suspension in well of ELISA plate by keeping volume upto 200 µl with PDB media. The same was performed for all dilutions; three replications of the experiment were followed for each chemical. For positive control, water was added along with media and spore suspension. Readings of optical density (OD) at the wavelength of 600 nm were recorded; plates were incubated at 25±2ºC. After incubation, readings at different time points were taken by spectrophotometer (Abbas *et al*., 2013). Biocontrol agents like Similarly, BCAs like *Trichoderma harzianum*, *T. viride PsJN* bacteria and non-pathogenic *Aspergillus flavus* were screened on lentils-agar media plates at 25±2ºC as dual culture. *T. Harzianum* significantly inhibited the growth of *A. lentis* on artificial media plates and was further selected to use in management trials.

Sowing of Lentils genotypes: Field trials were conducted in the experimental area of the Department of Plant Pathology. The soil was tested with a hygrometer for moisture analysis. Soil analysis was done at the Institute of Soil and Environmental Sciences and found that soil loam with a ratio of sand, 40% silt, 22% and clay 38%. Twelve advanced lines, including commercially grown varieties, were collected from Pulses Research Institute, Faisalabad. Genotypes were sown in the field with the plant to plant distance of 6 inches and 1.5 feet row to row distance by following the RCBD layout in three replications. Genotypes were also grown in pots in six replications. Fungus A. lentis was isolated from soil samples as well as diseased plant samples prior to field trials. For natural screening of the genotypes were sown in soil containing pathogen as was confirmed during isolation of pathogen from soil. All other agronomic practices and field operations were routinely performed, and the disease index was recorded.

***Inoculation tests on plants*:** Artificial inoculum of A. lentis 105spores/ml was given at the basal part of plant near roots by drenching and to the aerial parts through sprayer when the age of plant was 4-6 weeks. The concentration of spores was recorded at 108 spores/ml with the help of a Haemocytometer. Mock treatment on control plants was done with distilled water.

***Physiological Parameters*:** Ten days after applying inoculum of A. lentis, BCA like *T. harzianum* and *Aspergillus flavus* in pots, different physiological parameters (shoot length, root length, number of seeds per pod and Pod length) and biomass (Relative water content and cell membrane stability) of the individual plant were recorded and analyzed by using respective formulas. Such as Ali *et al.,* (2011).

***Relative water content*:** Relative water content was measured for detached leaves by calculating from top leaves by following the equation RWC (%) = (FW – DW)/ (TW – DW) × 100. The fresh weight was calculated after the excision of

leaves and for the turgid weight (TW), leaves were soaked in water for 24 h at 4 ºC for rehydration under darkness. Dry weight was taken by placing leaves in the oven for 48 h at 80 ºC. Cell membrane stability (CMS) was determined based on the amount of electrolyte leakage from the leaf cells following Ali *et al*. (2011). In this procedure, 0.4 g of plant leaf material (0.5 cm diameter leaf discs) was washed with double distilled deionized water and placed in tubes with 20 ml of water. The material was incubated for two hours at 25 °C. For CMS 0.4 g of leaves were taken and washed in deionized distilled water and were put in 20 ml distilled water-electrolyte leakage at 25C for 2 hours and reading as electrical conductivity (L1) of the solution containing leaf material was taken. Samples were then autoclaved at 120 °C for 20 min and electrical conductivity (L2) was taken when the temperature went down to 25°C. The CMS was the mean percentage of the leaf sample and was recorded as CMS (%) = [(1-(L1/L2)] × 100.

RESULTS

Screening of Lentil was done under natural conditions and data were recorded and analyzed further. The results of the screening are given below. Lines 11507 and 11509 show the minimum number of seeds. Line 12505 showed the maximum pod number per plant, while line 12503 showed the least no. of the pod. Line 12503 showed the maximum shoot length, while line M-09501 showed the least height. Line 12503 showed the full root length, while line 12514 showed the least.

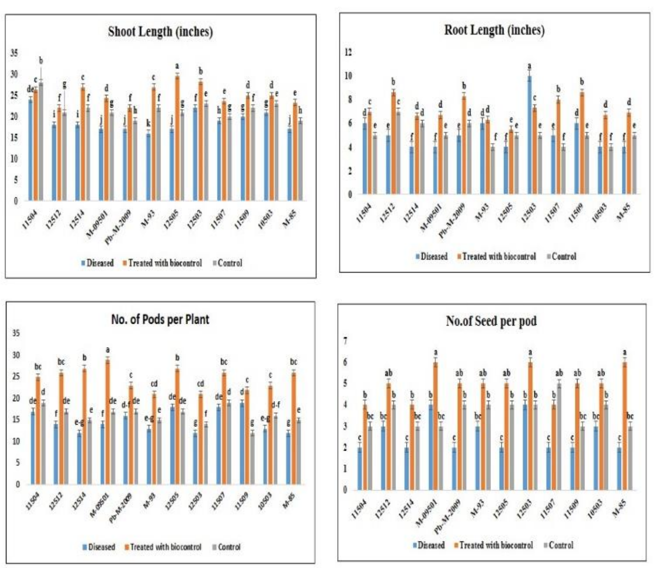

Figure 1. The bar graph shows the response of lines under natural inoculum of the *A. lentis* pathogen shoot length, root length, no. of seeds per pod and No. of pods per plant Graph 1. The bar graph shows the response of lines under artificial inoculum of the *A. lentis* pathogen Graph 3.

***Physiological parameters of Lentil treated with biocontrol*:** All lines show the maximum number of seeds per pod, while lines 11507 and 11509 show the least. Line 12503 shows the maximum length, while line 11509 shows the least. Line 12512 showed the maximum root length while line 11504 showed the least. Line 12505 showed the maximum shoot length while the line 12514 shown the least height.

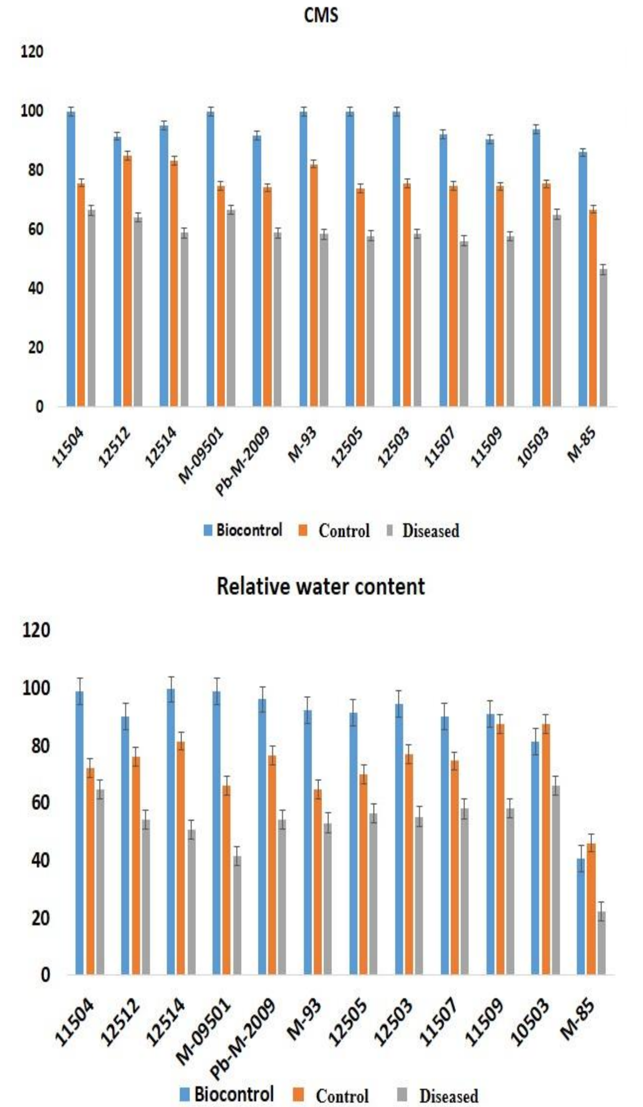

Figure 2. Cell membrane stability and relative water content shows that plants treated with biocontrol had enough resistance towards membrane stability while plants with disease has less resistance.

***Chemical control*:** Results showed that all chemicals are best at level 1 because of the high fungicide concentration. At the same time, all fungicides performed well at levels 4 and 5 while dolomite and difenoconazole did not show 50% results.

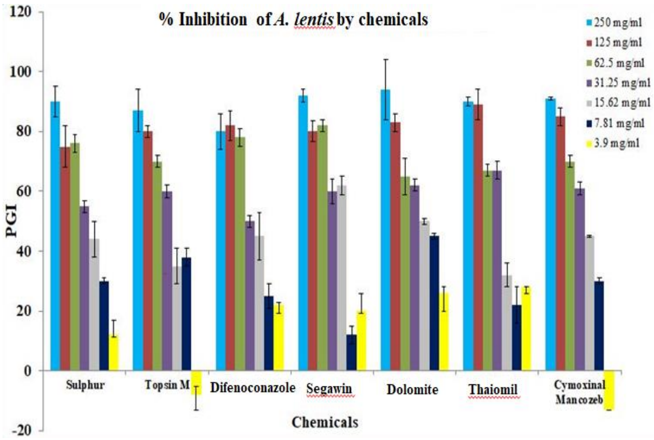

Figure 3. The bar graph shows the response of fungicide levels against the *A. lentis* pathogen.

DISCUSSION

Chaudhry *et al.* (2007) screened 196 lentil germplasm lines/cultivars for resistance against wilt and found no single line immune or highly resistant. Germplasm lines/cultivars for resistance against blight and found not a single line immune or highly resistant. Lentil screening against Ascochyta blight revealed that the disease's incidence and severity were high in the field. One of the reasons might be that crop often has the chance of disease escape, as the wilt disease is temperature dependent and the level of inoculum may vary at different places. Our results indicate that resistance in lentil germplasm to Ascochyta blight is not uncommon. In a greenhouse, Ayyub, (2001) evaluated 101 lentil lines for blight resistance; five were resistant, six exhibited moderate susceptibility, 13 were susceptible, and 74 were highly sensitive. Similarly, among 101 lines, nine lines could not show any expression of the disease symptoms under field conditions. In contrast, eight lines behaved as resistant and seven as moderately resistant. All the remaining lines were found to be susceptible to highly susceptible. Iqbal *et al.* (2005) reported resistant sources against Ascochyta blight in the lentil germplasm originating from national and international research institutes. He identified 14 lentil lines having resistance against wilt at the seedling stage, but no line was found to be resistant at the reproductive stage. Ayyub *et al.* (2003) reported a high level of resistance in lentil germplasm originating from different sources. Our findings regarding genotype screening are not different from the reports mentioned above; additionally, the effect of different fungicides validates previous reports. Trichoderma species are well recognized as soil-managing fungi against various soil-inhibiting fungal pathogens. (Schuster and Schmoll, 2010). Trichoderma is commonly used as a biocontrol agent due to its antimicrobial properties, including parasitism and competition. (Sivakumar, 2001). Trichoderma can produce various compounds like enzymes, proteins and antibiotics to manage pathogenic fungi. (Al-Taweil *et al.,* 2009; Vinale *et al.,* 2008). Aroosa *et al.* (2012) tested 14 chemicals against the Fusarium wilt of tomato and found a 20-80% growth reduction of *Fol* under in-vitro conditions.

***Conclusion*:** The present study was conducted to find an effective biocontrol agent against fungal pathogen of Lentil. Results indicate that these biocontrol agents not only help to deal with disease but also increase vegetative growth and yield of the crop. Results also give effective fungicidal dose with minimum chemical concentration, making it eco-friendly.

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